

PCR Troubleshooting- Part 1

“No Bands”

By Matt Bernstein- Technical Support

While the days of mineral oil and 2-minute ramp times are almost entirely a thing of the past, failed PCR is still as much a presence as it ever was. And even though the technology out there now is greater than ever, with more labs doing PCR on a regular basis and, at that, on rarer message transcripts and larger and larger genomic products, the mere opportunities for failure to occur are greater than ever too.

When technicians “fail” at PCR they usually refer to getting no product(s) on their ethidiums. Of course other examples of PCR failure can include getting the incorrect size of product, extraneous bands, or inconsistent results. This particular article will present some possible causes and solutions to the first and perhaps most common PCR failure- no bands at all on the ethidium. In the months to follow, articles on the other types of failures will be presented.

No Bands

Causes for no bands on a PCR can range from forgetting an ingredient in the reaction mix all the way to absence of the target sequence in your template DNA. Because there are so many possible causes for no bands from PCR, this article will attempt to present the most likely causes and most easily examined causes first before addressing less likely or more expensive ones. (Please see ["Help! My Protocol Isn't Working!"](#) for general strategies on protocol troubleshooting.)

1. Verify that you have not forgotten any reagents in your “Master Mix”.

There are two good ways to solve this. The first one is to make a checklist similar to this one:

- ☐ H₂O
- ☐ Buffer
- ☐ dNTP
- ☐ MgCl₂ ** Note- your buffer may already have MgCl₂ included
- ☐ Primer 1
- ☐ Primer 2
- ☐ Taq
- ☐ Template DNA

(Note that although these reagents don't have to be added in order, it is no coincidence that the template is listed at the end. Adding the template last helps prevent DNA contamination of your stocks during the preparation.)



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The second and most reliable (not to mention most convenient and consistent) way to prevent mistakes is to use a PCR pre-mix such as [Biomix](#). The low cost and high reproducibility of Bioline pre-mixes is one of the best preventative approaches you can take in your PCR. Only template and primers need be added which drastically reduces time, labor, and errors while increasing reproducibility. This is also your best approach to relative quantitative PCR applications.

2.. Concentrate on your Concentrations!

It is possible that you are not forgetting anything in your mix, but perhaps what you are adding is at the wrong final concentration. The following are recommended working concentrations for each reagent:

dNTPs: approximately .2mM. Be aware that too much dNTPs will actually have an inhibitory effect on your PCR! Our dNTPs can be found [here](#).

MgCl₂: Approximately 1-3mM. Though it is not likely that a 1 or 2 or even 3mM difference will prevent you from getting bands at all, it is likely that too much magnesium will result in smearing or extraneous bands. If you have at least 1.5mM magnesium, it is unlikely that increasing the concentration will suddenly produce your bands.

Primers: Approximately .5μM. Again, it is unlikely your primer concentration is to blame for complete absence of bands. Even as little as .1μM primer will be sufficient for most PCR.

Template DNA: While theoretically only one molecule is needed for amplification, realistically for a typical 25 or 30 cycle PCR this may not be sufficient. On the other extreme, using several μg of template may inhibit PCR because it could bind all your primers.

Enzyme: Generally 1-5U per reaction is commonly used, however I have had more than one customer inform me that they have been successful with as little as 0.1U of [Biolase](#) in 20ul reaction volumes.

3. Bad Reagents or Strategic Problem?

Usually the first thing researchers do is blame a faulty enzyme or reagent when an experiment fails but with PCR this is actually less likely to be the cause for failure. More often deeper internal problems such as primer design, thermocycler parameters, or nonspecific binding to other template sequences are the cause. Besides, DNA Polymerase is very stable and of course your primers, template and buffer are even more stable. The most likely suspect as far as reagents go would be the dNTPs. Making a habit of aliquoting the dNTPs to

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prevent freeze-thaw cycles and buying in smaller quantities if you don't do PCR very often are good practices. After that, a miscommunication on whether your buffer has $MgCl_2$ is quite likely. Without Magnesium, your polymerase will not function.

To most efficiently find if you have a reagent problem, you should do the following: find another researcher who's PCR is working (and who uses identical thermocycler parameters as your protocol) and ask him to make a PCR reaction using your template and primer as well as a control reaction with his template and primer- all using only his reagents. You should do the same exact thing using only your reagents. Run all four samples in the same PCR machine and the results will tell whether you have a reagent problem (his PCRs both work while yours both fail) or an internal strategy failure (only the control reaction works for both of you). If there is a suspected reagent problem, time would be saved in isolating the faulty reagent by making several PCR reactions with each one using a single substituted reagent from your colleague. Most likely the reaction using the colleague's (control) dNTPs will be the successful one.

One other point that fits under "bad reagents"- many researchers use TE buffer (10mM Tris, 1mM EDTA) to resuspend their DNA preparations. Because EDTA is a powerful chelating agent, it is possible it can bind your Mg and thus inhibit your PCR. Using only 10mM Tris or even just plain water is a better PCR template storage buffer than TE. Also be aware that many chemicals used in the very preparation of DNA (Phenol, Chloroform, alcohol, detergents) can strongly inhibit PCR and therefore if they are carried over into the final product it may cause your PCR to fail or slow considerably. DNA preparations that use a minimum of such reagents such as our line of [DNA Extraction Kits](#) are optimal for creating PCR templates.

4. Verify that your Target Sequence is pure, present, and compatible with your primers.

If you are at this stage, the good news is it's likely your reagents are functioning but the bad news is this is where things start to really get frustrating. Although you or your PI has certainly researched the target DNA and primers to be sure they match, no cost is involved in this step so it ranks as one of the first things you should double check when steps 1-3 verify there is indeed an internal strategy problem. If you have verified that your template is of sufficient purity (phenol, alcohol, EDTA, detergents, and other reagents carried over from DNA extraction will strongly inhibit PCR) then it's time to look at your strategies under the microscope. A common mistake when doing PCR off genomic DNA is that introns may be present between the primer sites which, if long enough, will result in incomplete product and thus no amplification.

It's also possible one or both primers are matching homologous sequences far away from the target sequence- maybe even on completely different

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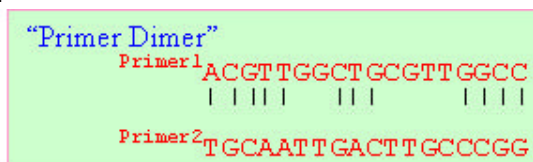
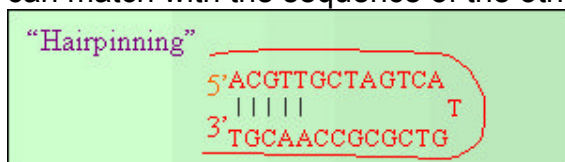
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chromosomes. Checking your primers in the [NCBI BLAST](#) database to make sure there are no other homologous sequences in your species is a good idea. (If you are not familiar with NCBI's BLAST you really should check it out. It's one of the most useful tools in the world for lab technicians doing genetics work and it's free! If you need help using BLAST, please [contact me](#) and I'll be glad to help get you started).

Time spent double and even triple checking your intended target sequence would be extremely wise at this point because it only gets tougher from here on.

5. A Primer on Primers

The next step after looking at your target DNA thoroughly is to examine your primer selection/strategy. The first thing to do is double check that you drew-up the primers correctly (1st primer is identical to the target's 5'→3' first strand sequence and second primer is a reverse complement of the target's 5'→3' second strand sequence). The other thing to do as far as primer sequence is verify that there will be no chance for hairpinning (primer loops on itself because of compatible sequences within the primer) or primer-dimerization (each primer can match with the sequence of the other).



While most primer design programs generally take care of things such as primer hairpinning and dimerization, they do not search Genbank as mentioned earlier to verify that the primer sequences are unique to the intended target sequence. Also, when it comes to primer selection, verify the primers have less than 60% GC content (I always try for about 45% max). Too high of a GC content can lead to nonspecific primer binding on other sequences or cause hairpinning/dimerization. As far as primer size, yes, size matters. About 20bp is the usual range for most PCR. Any less than 15bp and you risk having nonspecific binding of the primer to extraneous sites (similar to the way random hexamer primers work), as well as resulting in too low a Melting Point (MP) for practical PCR parameters. More than 30bp and you'll also risk nonspecificity because there is a higher chance that some sequences within the long primer will match to extraneous DNA sequences. Other facets on primer design and strategy will be covered next month in "Troubleshooting PCR Part II: Nonspecific Bands".

6. Welcome To The Machine!

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The final troubleshooting step is to see if you have a problem with your PCR machine parameters- or even a problem with the machine itself. Needless to say, if anyone else is using the machine with success, it is safe to assume the machine is functioning properly. The likelihood of mis-setting the parameters is far more likely than the possibility the PCR machine is indicating correct function on the control panel while not actually functioning properly at the block.

The two most important things to consider for setting the parameters for any PCR is the melting temperature of your primers and the length of the expected product.

For about 95% of my PCRs I actually used the same exact program. 4' Hot Start at 95c followed by 25-35 cycles of 1'@95c, 1'@55c, 1'@72c finished with a 5'@72c extension.

Of course, one of the tricks was I always chose primers with GC contents and melting temps to fit these parameters. To *roughly* estimate the melting temperature of your primers, you can use the following formula:

$$T_m = 81.5 + .41(\%GC) - 675/N - \%Mismatch$$

(T_m = Melting Temperature, N = number of bases in primer)

Primer melting temps between 45c and 60c are generally recommended, and these temperatures are used to estimate your annealing (middle step) temperature. Usually people estimate high on the annealing parameter and work down from there but, if you are having problems with no bands at all on your PCR, you may want to take the opposite strategy- start with a really low annealing temperature such as 37c and work up from there. If at 37c annealing you are still getting no bands at all then there is clearly a problem which has either already been mentioned earlier or the length of your PCR product may not be adequately accounted for in your PCR program.

The length of most researchers' PCR products is between 200 and 500bp, but certainly doing PCR on 24Kb products is not at all out the question. For most PCR, an extension time (the third setting) of 72c for 1 minute per Kb is more than sufficient to ensure a complete product. If you are only allowing, say, 30" extension for a 2Kb product, it is possible this is insufficient time for completing the product and you will not generate new products which will cause no bands on your PCR. As in the setting of the melting temperature parameters, it may be wiser to overestimate the extension time in an effort just to get bands to start appearing and then work down to a more convenient extension time from there.

A final PCR machine strategy would be to include a "Hot Start" in your program. Usually a hot start is used to prevent extraneous bands in your PCR but it definitely can be a help in some circumstances that may prevent bands from forming altogether. To do a hot start, there are two methods. The "old school" way is to add everything to each PCR tube (or well) except the DNA Polymerase and then bring the reactions to 95c. Then the polymerase is added to each sample at 95c. The idea is to prevent unwanted polymerase activity during the

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time the samples are heating up from room temperature to 95c. The problem with this method is it is very laborious for many samples, it's inconsistent (not all samples have the polymerase added at the same time) and it allows for horrible pipetting errors which would make procedures such as qPCR completely unreliable (adding only .5 or 1ul to each sample will cause great variability in the actual amount added per sample.) However, using a "Hot Start" polymerase such as our [Immolase](#) solves these issues easily because polymerase activity is suppressed until the denaturing temperature is reached. (Incidentally, if you need a high quality easy to use PCR machine, click [here](#).)

7. Other ideas

If by now your absence of bands from your PCR still hasn't been resolved, there are a couple of other things you can try:

First- switching to a premium polymerase such as [Accuzyme](#) or [Bio X-Act](#) may help. These are specifically designed for certain difficult templates and reactions and may be able to help with your application. My personal recommendation however would be [Biolase Diamond](#)- particularly if your template has a very high GC content and is therefore causing problems with denaturing. [Biolase Diamond](#) will often work in applications where "normal" polymerases cannot function. (If you do Mouse Tail screens by PCR, you might want to [ask me](#) about a simple two-step protocol for digesting/screening with results in about 6 hours –but only 10 minutes of hands on work!) Incidentally- be advised that Accuzyme is PFU based and will leave blunt ended products and not "A" overhangs so you will be unable to use the product in a "T" vector ligation.

You may also consider switching to our premium [PCR tubes, strip tubes, or plates](#). Our [Thinwall PCR tubes with Domed Caps](#) are ideal for troubleshooting your problem because the thin walls allow optimal thermotransfer and the domed cap ensures the tube is snugly fit into the block and the heated lid contacts the cap firmly. The use of tubes instead of plates can be useful in troubleshooting because the same reaction can be tried in different PCR machines to test completely different programs at the same time.

Sometimes just trying a different primer set can solve the problem. Even though the "mathematics" may all seem to work out on your primer selection, sometimes just using primers in different regions will "mysteriously" solve the problem. Indeed I have found this to be the case for me in the past on more than one occasion.

Still not getting any bands on your PCR? I won't give up if you won't. Call me at 800-227-9997 and ask for "Matt" or [e-mail me](#) and I'll be more than eager to listen to your application and protocol to help you get your PCR working as soon as possible.